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NATIVE FORM OF ENDOTHELIN RECEPTOR IN HUMAN PLACENTAL MEMBRANES

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SUMMARY. Little is known about the native form of endothelin receptor. To clarify its functional and structural properties, we solubilized the receptor from human placenta in an active form using mild detergents CHAPS and digitonin and showed that it is able to bind ^{125}I-endothelin-1 in a specific manner, with a pH optimum between 6 and 8 in contrast to a reported pH optimum of 4. The molecular weight of the receptor was estimated as 340,000 by gel filtration of the solubilized membrane in the presence of 0.2% (w/v) digitonin. When the solubilized membranes were labeled with ^{125}I-endothelin-1 prior to gel filtration, the radioactive ligand also migrated in the position corresponding to a 340 kDa protein. These results indicated that the native form of endothelin receptor in human placenta is a 340 kDa protein.

The action of the newly discovered vasoconstrictor peptide endothelins (1) shows a unique dependence on extracellular calcium ion for its physiological action. This interesting property attracted the attention of researchers. Specific binding of ET was demonstrated in various types of cells and tissues. The presence of specific binding proteins for ET was demonstrated in chick cardiac membranes (2), rat renal glomeruli (3), rat mesangial cells (4), and human placental membrane fractions (5). In these studies the binding proteins in membranes were labeled with ^{125}I-ET-1 by cross-linking methods, solubilized with the strong detergent sodium dodecyl sulfate (SDS), then subjected to electrophoresis in the presence of SDS. Controversy exists with respect to the molecular weight and properties of the binding site. More rigorous and extensive characterization of the endothelin receptor required for the clarification of its biochemical properties and the controversies demands the use of the native and biochemically active form of the receptor solubilized by a mild detergent treatment.

Here we report the solubilization of ET binding activity from human placental membrane fraction and its binding properties to ^{125}I-ET-1. The

The abbreviations used are: CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, ET: endothelin, PMSF: phenylmethylsulfonyl fluoride, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, EGTA: ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid.

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solubilized binding protein showed a greater molecular size than the binding subunits detected by cross-linking followed by SDS-gel electrophoresis.

MATERIALS AND METHODS

Materials. ET-1 was radioiodinated with [^{125}I]NaI (New England Nuclear) and the monoiodinated ET-1 was purified by HPLC with a Vydac C_{18} reverse phase column as described previously (5). The specific activity of ^{125}I-ET-1 was approximately 2,000 Ci/mmol. CHAPS and bis(sulfosuccinimidyl)suberate (BS^3) were purchased from Pierce Chemical Co. Leupeptin, aprotinin, PMSF, bacitracin, pepstatin, heparin (4-6 kDa), poly-L-lysine (22 kDa) and digitonin were obtained from Sigma Chemical Co. Bio-Gel A-1.5m (200-400 mesh) and marker proteins for gel filtration were from Bio-Rad. ET-1 was from Peptides International, Inc. All other reagents were of analytical grade.

Binding Assay. Binding assay was carried out according to the previous report (5) with slight modifications. Since ET-1 receptor forms aggregates readily and does not require divalent metal catious such as $MgCl_2$, $MnCl_2$ and $CaCl_2$ for stabilization or ligand binding, as shown below, we added 0.05% (w/v) Triton X-100 to the binding assay buffer to prevent aggregation and omitted $MgCl_2$. Briefly, 0.2 ml of binding assay buffer containing 30 mM HEPES (pH 7.5), 0.15 M NaCl, 0.5 mg/ml bacitracin, 0.4 mM PMSF, 1 mg/ml bovine serum albumin, 0.05% (w/v) Triton X-100 and CHAPS-solubilized membrane fraction (30 µg of protein) was incubated with 75 pM ^{125}I-ET-1 for 2 h at 4°C. Nonspecific binding was determined in the presence of 125 nM nonradioactive ET-1. The receptor-^{125}I-ET-1 complex was separated from free ^{125}I-ET-1 by filtration using a glass fiber filter (6).

Gel Filtration of Solubilized Membrane Fraction. Membrane fraction from human placentas was prepared and solubilized with 0.75% (w/v) CHAPS as described previously (5). To the solubilized membrane fraction, digitonin was added to a final concentration of 1% (w/v), then 0.25 ml of the solubilized fraction (containing 1.25 mg of protein) was applied to a Bio-Gel A-1.5m column (1 x 48 cm) equilibrated previously with 25 mM HEPES (pH 7.5) containing 0.15 M NaCl, 3 mM EDTA, 1 mM EGTA, 0.25 mg/ml bacitracin, 3 µg/ml leupeptin, 0.4 mM PMSF, 2 µg/ml aprotinin, 3 µg/ml pepstatin and 0.2% (w/v) digitonin. The flow rate of the column was approximately 4 ml/h, and 0.68 ml fractions were collected. Aliquots of 0.05 ml were used for measuring ^{125}I-ET-1 binding activity in the presence or absence of 125 nM unlabeled ET-1.

Binding and Cross-linking of ^{125}I-ET-1 Receptor. An aliquot of the membrane fraction solubilized with CHAPS (containing 0.75 mg of protein) was incubated with 400 pM ^{125}I-ET-1 in 0.18 ml of binding assay buffer in the presence or absence of 600 nM unlabeled ET-1 for 3 h at 4°C. After incubation, 20 µl of 10% (w/v) digitonin was added, then the sample was immediately applied to a Bio-Gel A-1.5m column as described above. For cross-linking experiment, the reaction mixture was further incubated with 0.8 mM BS^3 for 25 min at 4°C after binding of ^{125}I-ET-1 to solubilized receptor, and subsequently quenched by adding glycine (10 mM final). The sample was treated with 1% (w/v) digitonin, then analyzed by gel filtration as described above.

RESULTS

Specific binding of ^{125}I-ET-1 to the solubilized membrane fraction was studied under the various conditions as shown in Table I. Divalent metal ions such as Mg^{2+}, Mn^{2+} and Ca^{2+}, or EDTA did not alter the ET-1 binding activity. These divalent cations did not affect ^{125}I-ET-1 binding to intact membranes not

Table 1. Effects of various conditions on the ^{125}I-ET-1 specific binding to solubilized particulate fraction

Condition		%Control	Condition		%Control
Control		100	EDTA	(1 mM)	102
			$MgCl_2$	(10 mM)	101
	(1 μg/ml)	98	$MnCl_2$	(10 mM)	99
Heparin	(10 μg/ml)	100	$CaCl_2$	(10 mM)	104
	(100 μg/ml)	96			
				(0.2 M)	100
	(0.5 μM)	101	NaCl	(0.5 M)	97
Poly-Lys	(2.0 μM)	101		(1.0 M)	92
	(10 μM)	102			

solubilized by the detergent. Addition of 10 mM each of divalent cation to binding assay buffer resulted in 105 (Mg^{2+}), 97 (Mn^{2+}) and 96 (Ca^{2+}) % binding activities. Sodium chloride also showed no effect up to 1.0 M. Polyanionic (heparin) or polycationic (poly-L-lysine) substance showed no effect on the binding of ^{125}I-ET-1. The effect of pH on the specific binding was investigated (Fig. 1). The binding activity showed a symmetrical bell-shaped pH profile with an optimum pH range between 6-8. Intact membranes (without detergent treatment)

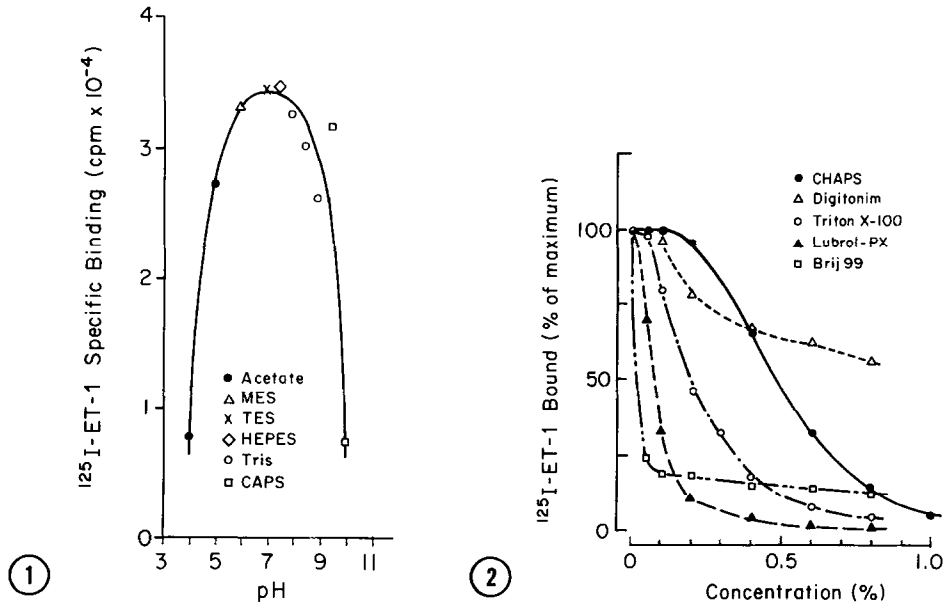

Fig. 1. Effect of pH on specific binding of ^{125}I-ET-1. CHAPS-solubilized membrane fraction (30 μg of protein) was incubated with 75 pM ^{125}I-ET-1 for 2 h at 4°C at various pH.

Fig. 2. Effect of detergent on specific binding of ^{125}I-ET-1. CHAPS-solubilized membrane fraction (30 μg of protein) was incubated with 75 pM ^{125}I-ET-1 for 2 h at 4°C in the presence of various detergents.

were also subjected to similar analysis. It also showed a similar pH-profile for the binding of ^{125}I-ET-1. Various detergents partially inhibited the ^{125}I-ET-1 binding to the solubilized membrane fractions in dose-dependent manners (Fig. 2). Among them digiton showed less inhibition than any other detergents tested, and 56% of binding activity remained even at 0.8% (w/v). The concentrations of CHAPS and Triton X-100 causing half-maximal inhibition were approximately 0.5% (w/v) and 0.2% (w/v), respectively, while those of Lubrol-PX and Brij 99 were below 0.1%.

In order to determine the native molecular weight of ET-1 receptor, the membrane fraction solubilized with CHAPS was further treated with 1% (w/v) digiton prior to gel filtration, then applied to a Bio-Gel A-1.5m column as described under "MATERIALS AND METHODS". ET-1 specific binding activity was eluted as a single peak with a molecular weight of 340,000 (Fig. 3). The binding activity under the peak remained stable for several days at 0°C. If solubilized membrane fractions were labeled with ^{125}I-ET-1 by cross-linking

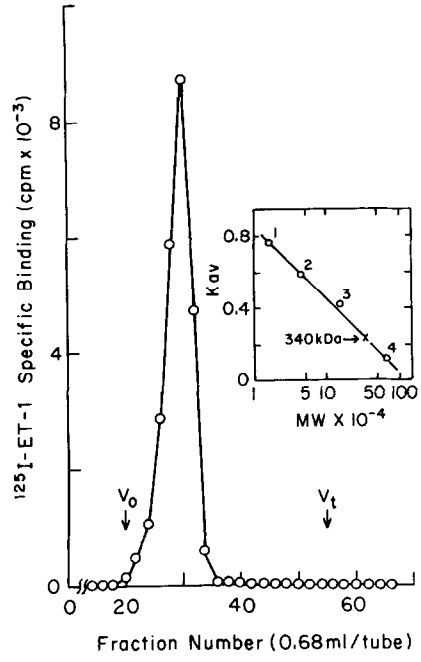

Fig. 3. Gel filtration of solubilized membrane fraction. CHAPS-solubilized membrane fraction (1.25 mg of protein) was treated with 1% (w/v) digitonin and then applied on a Bio-Gel A-1.5m column (1 x 48 cm) in the presence of 0.2% (w/v) digitonin. Fractions were assayed for specific binding of ^{125}I-ET-1 as described under MATERIAL AND METHODS". The inset shows the molecular weight determination of ET-1 receptor. Standard proteins (o) included were 1, myoglobin (17 kDa), 2, ovalbumin (44 kDa), 3, γ-globulin (158 kDa), and 4, thyroglobulin (670 kDa). The molecular weight of ET-1 receptor (x) was estimated to be 340,000 . Vo and Vt indicate the elution positions of blue dextran and cyanocobalamin, respectively.

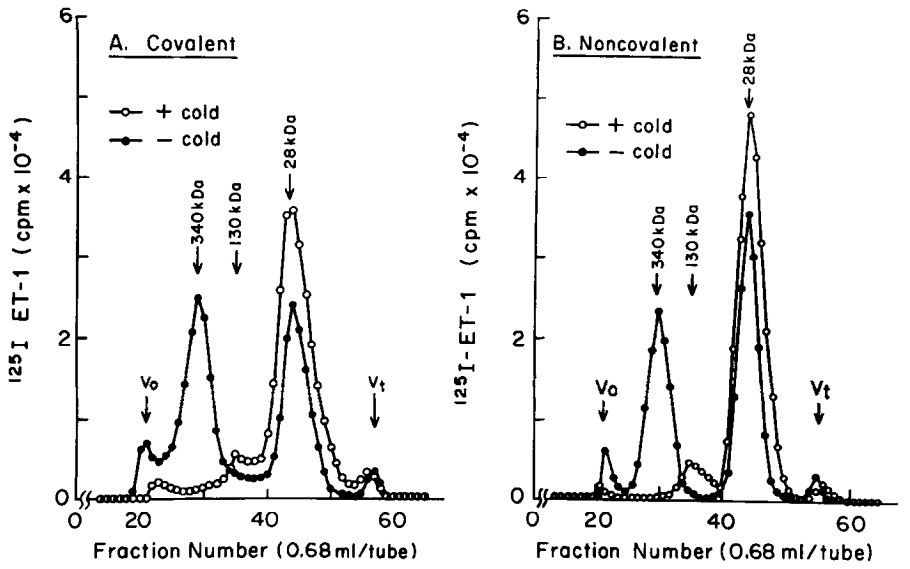

Fig. 4. Gel filtration of ^{125}-I-ET-1-receptor complex. CHAPS-solubilized membrane fraction (0.75 mg of protein) was incubated with 400 pM ^{125}I-ET-1 in the presence (o) or absence (•) of 600 nM unlabeled ET-1 and was reacted with (A) or without (B) BS^3. The sample was further treated with 1% (w/v) digitonin and then subsequently subjected to gel filtration as described under "MATERIALS AND METHODS".

using BS^3 prior to gel filtration, two major peaks were identified as shown in Fig. 4A. The molecular weights of the two peaks were 340,000 and 28,000, respectively. When ^{125}I-ET-1 binding was carried out in the presence of excess cold ET-1 prior to the chromatography, the 340 kDa peak disappeared. On the contrary, the 28 kDa peak was not abolished by excess cold ET-1, furthermore, 130 kDa peak increased significantly, indicating that 130 kDa and 28 kDa proteins were nonspecifically labeled with ^{125}I-ET-1. The same result was obtained from gel filtration of noncovalent complex between ^{125}I-ET-1 and receptor (Fig. 4B). These results indicate that the native molecular form of ET-1 receptor on human placental membranes is a 340 kDa protein. SDS gel electrophoresis of the solubilized membrane fractions labeled with ^{125}I-ET-1 (cross-linked) gave a 32 kDa band as shown previously (5).

DISCUSSION

The present results indicate that the receptor for ET-1 can be solubilized in a stable and functionally active form. To date many ET binding studies have been demonstrated with intact membranes. The present results open the way for the isolation and characterization of solubilized ET-1 receptor.

Treatment of the placental membranes with 1% (w/v) digitonin solubilized ET-1 receptor completely which could be observed as a sharp 340 kDa peak upon

gel filtration. Confirmation of 340 kDa protein as specific ET-1 binding protein was also obtained by the solubilized membranes prelabeled with ^{125}I-ET-1 with or without cross-linking reagent, BS^3 (Fig. 4A and 4B, respectively). On the other hand 0.75% (w/v) CHAPS produced incomplete solubilization with 65% of the binding activity eluting at the position of void volume and a small amount of binding activity eluting as a broad peak with a molecular weight range of 420,000-350,000.

The binding of ^{125}I-ET-1 to the solubilized membranes did not require EDTA or divalent cations such as Mg^2, Mn^{2+} or Ca^{2+}. These observations are in contrast to the previous reports that Mn^{2+} stimulates the binding of ^{125}I-ET-1 to rat renal papillary membranes (3) or human placental membranes (7). The lack of stimulation of the binding by Mn^{2+}, Mg^{2+}, Ca^{2+} or EDTA was confirmed with intact human placental membranes as well as solubilized membranes. The reason for the discrepancy is not clear. The binding of ^{125}I-ET-1 to rat papillary membrane receptor in the absence of a detergent was reported to be optimum at pH 4.0 (3). Present studies using detergent or intact human placental membranes showed optimal binding at a neutral pH range. Again, the reason for the discrepancy is not clear.

Miyazaki et al. (8) have shown that ET-1 receptor in chick heart differed from that for dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel based on the molecular weight which was estimated by ultracentrifugation. The molecular weight of ET-1 receptor in chick heart was greater than that of immunoglobulin. This result is in support of the result obtained in the present study. Previously we have identified 32 kDa protein on human placental membranes as ET-1 binding protein by chemical cross-linking procedure (5). A similar subunit was obtained in the solubilized membrane in the present studies. Taken together these results indicate that 340 kDa ET-1 receptor on human placental membranes are composed of subunits including 32 kDa protein as ET-1 binding subunit.

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